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ORIGINAL ARTICLE

Heterologous expression of pepper mild mottle virus coat protein encoding region and its application in immuno-diagnostics

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Abstract Pepper mild mottle virus (PMMoV), a tobamovirus of family *Virgaviridae* affects the quality and quantity of Capsicum. PMMoV is highly contagious, capable of transmitting through infected seeds and soil. Symptoms are more severe when crop is infected at young stage but remain unnoticed when infection takes place at maturity. Therefore, cost effective diagnostic techniques are required for timely and accurate detection of virus. In present study, coat protein encoding region of PMMoV-HP1 isolate was cloned into expression vector system, *pET28a* and expressed in BL21, a protease deficient strain of *Escherichia coli*. The PMMoV-HP1 pathotype was identified as PMMoV-P_{12} on the basis of coat protein amino acid sequence analysis in our previous study. The overexpression of recombinant coat protein of 26 kDa, corresponding to the expected 6X Histidine tag fused recombinant protein was purified using Ni–NTA columns from insoluble fraction. For antisera production, the purified recombinant protein was dialyzed $\sim$ 24 h to remove urea and then used for raising polyclonal antisera. The specificity and sensitivity of antiserum obtained was demonstrated using different dilutions of antiserum for western blot assay and direct antigen coating enzyme linked immunosorbent assay (DAC-ELISA). In Western blot assay, the test antiserum reacted strongly both with PMMoV-CP in purified protein and native CP in crude sap from PMMoV infected pepper plants, whereas no reaction was observed with healthy plant sap. In DAC-ELISA antiserum dilution up to 1:1000 was capable of detecting the virus in infected sample. The absence of any cross reactivity of test antiserum was confirmed against tobacco mosaic virus, cucumber mosaic virus, tomato spotted wilt virus, pepper veinal mottle virus, potato virus Y and tomato yellow leaf curl virus antigen, known to infect capsicum.

Keywords PMMoV · Coat protein · Capsicum · Western blot assay · DAC-ELISA

Introduction

Capsicum annuum L. var. *grossum* Sendt (Bell pepper or Sweet pepper), cultivated across the world under various climatic and environmental conditions [20] is an important spice and vegetable crop. *Capsicum* spp., originated in Mexico, South Peru and Bolivia [22, 42, 53], in India, popularly known as "Shimla Mirch" is one of the premier vegetable crops being cultivated in open as well as protected conditions. As per the records of National Horticultural Board, the total area and production of capsicum in India is 24,000 ha and 306,000 MT, respectively (http://nhb.gov.in/statistics/State_Level/2016-17(Final). The production of capsicum is being impeded by many pathogens including viruses [46] which are important contributing factor for low produce yield and poor fruit quality [21, 28]. Capsicum is known to be infected by about 68 virus species from *Potyvirus*, *Carlavirus*, *Potexvirus*, *Tobamovirus*, *Tobravirus*, *Luteovirus*, *Tospovirus*, *Cucumovirus* genera, however, 20 virus species are reported to cause considerable damage to the crop [38]. Among these, Pepper mild mottle virus (PMMoV), a member of *Virgaviridae* family and genus *Tobamovirus*, is emerging as a major threat to the Capsicum cultivation across the globe [33, 37, 45, 48].

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Initially recognized as a strain of *Tobacco mosaic virus* (TMV) by Mckinney in 1952 [36] and cited as pepper strain of TMV in early literature. Wetter et al. [58] isolated the virus from TMV resistant peppers in Sicily, Italy and named it pepper mild mottle virus. Since then, this virus and its pathotypes has been intercepted from number of capsicum growing countries that are capable of breaking *L* allele mediated resistance [5, 7, 17, 19, 24, 55]. The occurrence of PMMoV from India was first time encountered by Sharma and Patiyal [49] in polyhouse grown capsicum of Himachal Pradesh (HP) situated in northern part of the country and genome of this isolate was determined by Rialch et al. [45].

PMMoV is a rod shaped virus with positive sense RNA genome of ~ 6.3 kb size [45]. PMMoV causes mild to severe symptoms on capsicum which includes mosaic on leaves and fruits, mottling, puckering of leaves, vein thickening, stunting, leaf upward cupping, fruit deformations [4, 45]. The virus is highly contagious capable of being transmitted through seed [9, 18] and soil [26, 54, 59]. PMMoV may initiate the disease through infected seeds, infected soil or through contact with the infected plant or via agricultural implements thus have potential risk to cause an epidemic. Moreover PMMoV produces mild symptoms which sometimes remain unnoticed in the field and become evident only at the fruiting stage [41, 45].

Though development of virus resistant transgenic plant varieties in the recent past is one of the most effective and viable option for the management of virus diseases but these crop varieties have very low acceptance among the farmers and consumers [16, 44]. In general, use of disease free planting material and other cultural practices becomes mandatory for the control of viral diseases. Therefore accurate, rapid, specific, sensitive, economic and high-throughput techniques are required for the detection of viruses as it is the foremost important step for crop management system [1].The most common and widely used techniques for detection of plant viruses in general and PMMoV in particular includes enzyme linked immunosorbent assay (ELISA) and reverse transcriptase polymerase chain reaction (RT-PCR) [10, 12, 27, 35, 51, 57]. However, molecular methods like RT-PCR require well equipped molecular biology laboratory, pathogen specific markers where as serological techniques are preferred methods for routine use and indexing of large sample sizes because of the comparative low cost involved [26, 52, 56]. To achieve sensitivity and specificity in serological assays, the requirement of good quality antibodies lays emphasis on the necessity of highly purified virus preparations. Hence raising of viral antigenic proteins through recombinant DNA technology provides an opportunity to get highly identical proteins to generate pure polyclonal antibodies (PAbs). The most common virus protein used in identification of most plant viruses is coat protein (CP) that build the capsid of plant viruses [8]. The viral capsid is composed of repeating subunits called capsomeres. For a given virus, the identical capsomeres have identical amino acid (aa) content and sequence but they are dissimilar for other viruses or even different strains of the virus. Therefore, use of bacterial expressed CP of plant viruses as immunogen is the novel technique to generate PAbs [39] due to the difficulties encountered during the purification of virus from infected plants and undesirable reaction in test animals due to the contamination of other virus or host proteins. Therefore present study aims at overexpression of PMMoV-HP1 (P_{12} pathotype) coat protein in heterologous host and its purification for the production of polyclonal antiserum for developing serology based low cost indigenous diagnostic kit.

Materials and methods

Sample collections, total RNA isolation, cDNA synthesis and RT-PCR

The PMMoV-HP1 (P_{12} pathotype) virus isolate [45] maintained on capsicum variety "California wonder" in greenhouse of the Department of Plant Pathology, CSK HP Agricultural. University, Palampur, India was used in this study. The total RNA was isolated from the PMMoV-HP1 infected bell pepper var. California Wonder plant using Trizol reagent following the manufacturer's instructions (Invitrogen). Total RNA isolated was subjected for cDNA synthesis using Verso cDNA synthesis kit following the manufacturer's instructions (ThermoFisher Scientific). The presence of PMMoV-HP1 in the sample was confirmed through RT-PCR using CP gene specific primers (CPF: 5′ CCAATGGCTGACAGATTACG 3′, CPR: 5′ CAACGACAA CCCTTCGATTT 3′) [45].

Construction of pET28a-CP system

cDNA synthesized from total RNA isolated of PMMoV infected plant was used to amplify CP region of virus. For overexpression, primer pair with built in restriction sites for *EcoRI* and *SalI* in forward and reverse primer respectively (F: 5′-ACGAGAATTCATGGCTTACACAGTTTCCA-3′, R: 5′-ATCGGTCGACGGAGCGGAGTTGTAGCCCAGGTGA-3′) were used. The RT-PCR was performed in 50 µl reaction containing 2 µl of cDNA template, 2 µl of each primers (10 mM), 5 µl of dNTPs mix (2 mM each), 5 µl of 10 X PCR buffer, 1.5 µl of 25 mM $MgCl_2$ and 0.5 µl of Taq DNA polymerase (5 U/µl). The final volume was adjusted using nuclease free water. The PCR conditions consisted of initial denaturation of 94 °C for 4 min

followed by 35 cycles of 94 °C for 15 s, annealing at 65 °C for 40 s and extension at 72 °C for 1 min with a final extension of 5 min at 72 °C. The PCR product was analyzed on 1.2% agraose gel stained with 2 µl of ethidium bromide (10 mg/ml) and eluted from agarose gel using gel extraction kit (GeNei) following the manufacturer's instructions. The eluted product was ligated in pGEMT-Easy vector (Promega), and transformed into DH5α strain of *E. coli*. The plasmid isolated from the transformed recombinant colony was subjected to double digestion with *EcoRI* and *SalI*. For cloning into pET-28a expression vector, vector pre-digested with *EcoRI* and *SalI* was ligated to fragment with respective restriction sites and then transformed into BL21 (DE3) strain of *E. coli*. The positive recombinant colonies were custom sequenced (Agrigenome Labs pvt. ltd, Kerala, India) with the universal T7-promoter and terminator primers and analyzed for any frameshift.

Over expression, purification of recombinant coat protein

For expression study, the recombinant clone containing p*ET28a*-CP was inoculated in 5 ml Luria berthani broth (LB broth) containing Kanamycin (100 mg/ml) and grown overnight at 37 °C at 170 rpm in an incubator shaker. One ml overnight grown culture was used to inoculate 500 ml of LB broth containing kanamycin and incubated at 37 °C at 200 rpm in an incubator shaker till $O.D_{600nm}$ reaches 0.6–0.8. The over expression of recombinant coat protein was induced using 1 mM isopropyl-thio-β-Dgalactopyranoside (IPTG) for overnight at 16 °C. The culture was centrifuged at 5000 rpm for 20 min at 4 °C and the pellet was suspended in lysis buffer B (100 mM NaH_2PO_4, 10 mM Tris, 8 M Urea, pH: 8.0 adjusted with NaOH). The lysate was incubated at room temperature for 20–30 min followed by centrifugation at 5000 rpm for 20 min. The pellet thus obtained was discarded and supernatant was retained. The level of induction was analyzed using this supernatant through 15% SDS-PAGE followed by gel staining with Coomassie brilliant blue 250. The expressed protein was purified using nickel–nitrilotriacetic acid (Ni–NTA) columns (Clontech) following the manufacturer's instructions. The purity of the purified protein was assessed using SDS-PAGE.

Solubility analysis of expressed protein

To check the solubility of target protein, an experiment was conducted where two buffers viz. native buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidazole, pH 8.0 using NaOH) and lysis buffer B (100 mM NaH_2PO_4, 10 mM Tris Cl, 8 M Urea, pH 8.0 using NaOH.) was used to resuspend the pellet from uninduced as well as induced culture. Both cultures were collected and pelleted down in similar way as described in the previous section. The pellet from the induced culture was resuspended in 1 ml of native buffer and incubated on ice for 20 min. To this lysate, 1 mg/ml lysozyme was added and incubated on ice for 20 min. The lysate was sonicated with 6 × 10 s with 10 s pauses at 200–300 W. The lysate was kept on ice all the time during sonication. The lysate was centrifuged at 10 000 rpm for 20 min at 4 °C. The supernatant (crude extract A, soluble protein) was taken in another tube and saved for analysis. The pellet obtained thereafter was resuspended in lysis buffer B (crude extract B). SDS-PAGE was performed as described in earlier section to analyze both A and B crude extracts.

Confirmation of 6x-His tagged recombinant protein

The production of recombinant protein was established through western blot assay. For blotting, protein samples were electrophoresed in 15% polyacrylamide gel under denaturing conditions and electroblotted on polyvinylidene difluoride (PVDF) (Novex, life technologies) membrane in Semi-Dry electroblotter (Peqlab) at 30 V for 3 h at room temperature. The blotted membrane was incubated overnight at 4 °C in blocking buffer (3% Bovine serum albumin, BSA) followed by incubation with 6x His-tagged Monoclonal antibody (Novagen^R) (1:1000) at room temperature for 2 h with gentle shaking. Following this, the membrane was incubated with goat anti mouse IgG alkaline phosphatase (ALP) conjugated antibody (1:5000) for 2 h at room temperature. After each step the membrane was washed with 20 ml of Tris-buffer saline-Tween (TBST, 20 mM Tris–HCl, 150 mM NaCl, 0.05% (v/v) Tween-20, pH 7.2) for 3 times at least. The 5-bromo-4-chloro 3-indolyl-phosphate and nitroblue tetrazolium (BCIP/NBT) solution (Sigma, ready to use) was added to the membrane after final wash and incubated under dark conditions to visualize the reaction.

Production of polyclonal antiserum

In order to solubilize the protein in insoluble fraction, the crude extract B was subjected to incubation at room temperature with gentle shaking for 30 min followed by centrifugation at 10,000 rpm for 20 min. The supernatant thus obtained was subjected to affinity column chromatography using nickel–nitrilotriacetic acid (Ni–NTA) columns (Clontech). The purified protein was quantified using Qubit 2.0 flurometer, dialyzed using 50 mM Tris buffer to remove the traces of urea for more than 48 h and then used for raising custom polyclonal antisera (Genei Laboratories Pvt. Ltd. (Bangalore, India). The antiserum was raised in

New Zealand white male rabbit. For primary immunization, the homogenized protein sample solution (2 mg) was mixed with 500 µl of freund's complete adjuvant (FCA) and administered to rabbits through subcutaneous routes. Further 5 booster doses mixed with 500 µl of freund's incomplete adjuvant (FICA) were given at 15 days interval. Blood was collected and coagulated at 37 °C for 1 h followed by centrifugation at 4000 rpm for 10 min. The supernatant was collected and stored at − 20 °C till further use.

Evaluation of polyclonal antiserum through western blot assay and DAC-ELISA

To evaluate the efficiency, sensitivity and specificity of obtained antiserum, western blot assay and DAC-ELISA was performed with different dilutions of antiserum. The virus was detected in purified expressed protein (346 µg/ml), PMMoV infected pepper leaves and healthy pepper leaves through western blot assay as well as DAC-ELISA. To prepare the crude extract from PMMoV infected and healthy pepper leaves, the leaves were crushed in buffer (1 g leaf tissues in 10 mL buffer) containing 0.24 g Tris, 0.80 g NaCl, 2 g Polyvinyl-pyrrolidine (PVP), 0.05% Tween 20, 20 mg KCl and NaN_3 in 1000 ml distilled water (pH 7.4). The homogenized mixture was centrifuged at 4000 rpm for 20 min at 4 °C and supernatant was collected and used in western blot assay. The expressed protein along with the extract from PMMoV infected and healthy pepper leaves was electrophoresed in 15% polyacrylamide gel under denaturing conditions and electro-blotted on polyvinylidene difluoride (PVDF) membrane. The blotted membranes were subjected to western blot assay as described in earlier section, using crude antiserum diluted in blocking buffer at 1:100, 1:500, 1:1000, 1:2000, 1:3000, 1:4000, 1:6000, 1:8000, 1:10,000 and 1:20,000 dilutions. The secondary antibody used was Goat anti-Rabbit IgG-ALP (GeNei) at 1:1000 dilution.

To perform DAC-ELISA, the sample was prepared by crushing the infected and healthy leaves in coating buffer (1 g leaf tissues in 10 mL buffer) (1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, 0.20 g NaN_3 with 2% PVP in 1000 ml distilled water, pH- 9.6). Firstly the purified protein, extract from PMMoV infected and healthy pepper plants were coated onto the polystyrene plate and incubated at 37 °C for 2 h. The plate was blocked with 3% BSA in PBS-T buffer for 1 h at 37 °C. After the blocking step, 100 µl of crude antiserum at dilutions viz., 1:100, 1:500, 1:1000, 1:2000, 1:4000 and 1:8000 was added and incubated overnight at 4 °C. After the overnight incubation, 100 µl of Goat anti rabbit IgG conjugated with ALP enzyme (GeNei, diluted to 1:1000 times) was added to plate and incubated for 2 h at 37 °C. Three washings with PBS-T (1 × PBS buffer + 0.05% Tween-20) for five minutes was given after each step. Finally the substrate para-nitrophenyl phosphate (pNPP) (Bioreba) was added and the plates were kept for incubation at room temperature under dark conditions for one hour or till the development of color. The reaction was stopped using 50 µl of 3.0 M NaOH and absorbance at 405 nm was recorded using MULTISKAN FC ELISA reader (Thermo Scientific).

Validation of polyclonal antiserum for PMMoV detection from leaf tissues and seeds

The specificity of polyclonal antiserum was further validated by detecting PMMoV from leaf tissues of 20 artificially inoculated capsicum plants at 1:500 and 1:1000 dilutions through DAC-ELISA. The cross reactivity of the test antiserum was tested against other bell pepper viruses viz., TMV, *Cucumber Mosaic Virus* (CMV), *Tomato Spotted wilt virus* (TSWV), *Pepper veinal mottle virus* (PVMV), *Potato virus Y* (PVY) and *Tomato yellow leaf curl virus* (TYLCV) at 1:500 and 1:1000 dilution. For detection of the virus from seed, the seeds harvested from the fruits of artificially inoculated plants kept in butter paper bags at 4 °C were used. The antigen was prepared by grinding the 10–12 seeds (∼ 55 mg) in 1 ml buffer followed by centrifugation at 5000 rpm for 5–10 min, the supernatant thus obtained was used as antigen in DAC-ELISA. The antiserum dilutions were same as described above.

Results

PCR amplification, and construction of p*ET*28a-PMMoVCP construct

The presence of PMMoV-HP1 was confirmed through RT-PCR where an amplification of ∼ 740 bp was obtained in infected samples (Fig. 1a). Further an amplicon of ∼ 500 bp corresponding to PMMoV CP region using primers with built in restriction sites of *EcoRI* and *SalI* obtained in RT-PCR (Fig. 1b) was cloned into pGEM-T Easy cloning vector. Insert released after *EcoRI* and *SalI* double digestion was ligated into p*ET*28a vector predigested with same restriction enzymes (Fig. 1c). The validity of ligation was established by colony PCR followed by DNA sequencing and NCBI-BLAST analysis of positive clones. The obtained sequence of 486 nucleotides corresponded to PMMoV-genome (Fig. 1d) at 5685–6154th nucleotide position. The BLASTn analysis confirmed the identity of sequence as PMMoV-CP with 100% similarity to the isolate PMMoV-HP1 (KJ631123.1) [45]. The obtained sequence was translated using ExPassy

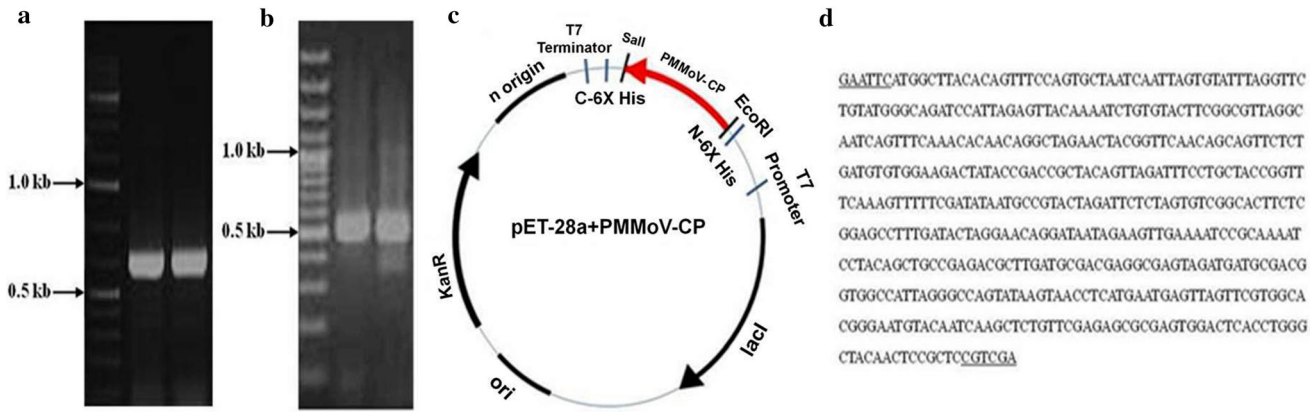

Fig. 1 **a** RT-PCR amplification of PMMoV-HP1 infected "California wonder" plants; **b** Amplification of PMMoV-CP gene using primers with built in restriction sites for *EcoR1* and *Sal1*; **c** A diagram showing PMMoV-CP cloned in expression vector, pET-28a; **d** Nucleotide sequence of coat protein region of PMMoV cloned in pET-28a

Translate Tool and compared with the amino acids sequence of CP gene of PMMoV-HP1 isolate (KJ631123.1). The amino acids sequence of present pET-28a-PMMoVCP was 100% similar to PMMoV-HP1 isolate (Fig. 2). Thus these results confirmed the successful insertion of full length PMMoV-CP gene in pET-28a expression vector without any frameshift.

Overexpression, solubility and purification of recombinant protein

The induction of recombinant CP was confirmed using 15% SDS-PAGE where 1 mM IPTG was able to induce the overexpression of protein. Induced protein of ~ 26 kDa corresponding to predicted size was observed in SDS-PAGE whereas absence of same in the uninduced culture confirmed its overexpression (Fig. 3a). SDS-PAGE after protein purification through Ni–NTA affinity chromatography also revealed a single band of ~ 26 kDa molecular weight in the elution fraction. To check the localization of target protein, buffers viz., native buffer and lysis buffer B were used to suspend the pellet. Both A and B crude extracts were analyzed through SDS-PAGE (Fig. 3b). The SDS-PAGE analysis showed the presence of expressed target protein in the insoluble fraction. The recombinant protein expression was also established through western blot assay using 6x His-tag monoclonal antibody as primary antibody and goat anti mouse IgG ALP conjugated antibody. The presence of single band corresponding to molecular weight i.e. 26 kDa confirmed the overexpression of 6X His tagged protein in BL21 strain of *E. coli* (Fig. 3c).

Evaluation and validation of raised polyclonal antiserum

A total of 2.0 mg purified 6X-His tagged recombinant protein (346 µg/ml) quantified through Qubit 2.0 flurometer was used to immunize rabbit for production of polyclonal antiserum using custom services. The polyclonal antiserum thus obtained was subjected to western blot assay and DAC-ELISA for evaluating its specificity and sensitivity against the PMMoV using purified expressed protein sample; PMMoV infected plants with different dilutions of the antiserum. In western blot assay, the raised PMMoV antiserum reacted with PMMoV-CP in purified protein sample as well as crude sap extracted from PMMoV infected plants (Fig. 3d). The molecular weight of CP band observed in case of overexpressed protein and infected plant's crude sap was ~ 26 kDa and ~ 17 kDa, respectively. The molecular weight of PMMoV CP is 17.2 kDa and protein band of same size was obtained in case of infected plant's crude sap though a significant

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pET-28a+CP      MAYTVSSANQLVYLGSVWADPLELQNLCTSALGNQFQTQQARTTVQQQFSDVWKTIPTAT
HP-1            MAYTVSSANQLVYLGSVWADPLELQNLCTSALGNQFQTQQARTTVQQQFSDVWKTIPTAT
                ************************************************************
pET-28a+CP      VRFPATGFKVFRYNAVLDSLVSALLGAFDTRNRIIEVENPQNPTTAETLDATRRVDDATV
HP-1            VRFPATGFKVFRYNAVLDSLVSALLGAFDTRNRIIEVENPQNPTTAETLDATRRVDDATV
                ************************************************************
pET-28a+CP      AIRASISNLMNELVRGTGMYNQALFESASGLTWATTP
HP-1            AIRASISNLMNELVRGTGMYNQALFESASGLTWATTP
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Fig. 2 Alignment of amino acid sequence of pET-28a + CP with that of PMMoV-HP1 generated through multiple alignment tool clustalW

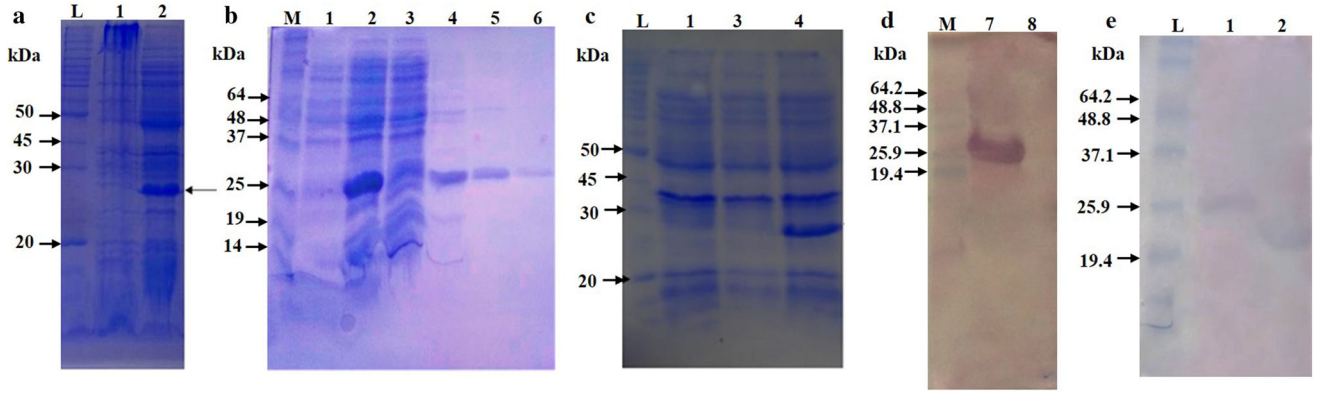

Fig. 3 15% SDS-PAGE presenting recombinant coat protein gene expressed using 1 mM of IPTG (**a**); recombinant protein purified through Ni–NTA affinity chromatography, Lane L: Benchmark unstained protein ladder (Fischer Scientific, Cat. No.: 10747-012) Lane 1: uninduced sample, Lane 2: induced sample, Lane 3: flow through fraction collected, Lane 4-6: three elution fractions collected separately (**b**); Solubility analysis of target protein. L: unstained protein ladder, Lane 1: uninduced sample, Lane 3: crude extract A (soluble protein fraction), 4: crude extract B (insoluble protein fraction) (**c**); Western blot analysis following electrophoresis in SDS-PAGE, electroblotting of purified recombinant protein using 6XHis-tag specific monoclonal antibodies Lane M: Benchmark prestained protein ladder (Fisher Scientific, Cat. Number: 10748-010), Lane 7- represents purified recombinant protein, Lane 8- represents the uninduced culture (**d**) and purified recombinant protein and crude extract of PMMoV infected plants using 1:100 dilution of polyclonal antiserum. L: Benchmark pre-stained protein ladder (Cat. No.:10748-010), Lane 1: purified recombinant protein, Lane 2: crude extract isolated from PMMoV infected plants (**e**)

difference in protein size was obtained in case of overexpressed protein and infected plant's crude sap due to lack of tag at C and N terminal of the native protein. The antiserum could detect the viral CP in purified protein sample up to 1:10,000 dilution; while in crude sap antiserum dilution of 1:8000 was able to detect the viral CP. No reaction was observed in case of total protein isolated from healthy pepper leaves. The antiserum did not show any non-specific reaction with infected plant tissues and purified virus.

In DAC-ELISA, the antiserum dilutions of 1:100, 1:500 and 1:1000 were able to detect the antigen in infected samples. The absorbance value at 405 nm of PMMoV infected plants ranged from 1.372 to 0.122 while in case of purified virus the values ranged from 2.610 to 0.901. The absorbance values for healthy samples remained lower than 0.340 at all dilutions used (Table 1). However, the absorbance values for purified protein were higher than the crude sap from PMMoV infected leaves at all the tested dilutions. The antiserum detected PMMoV in all the artificially inoculated capsicum plants but showed negative reaction with other pepper viruses (Table 2). Additionally, both the antiserum dilutions has also successfully detected PMMoV from seeds exhibiting OD_{405} values much higher than the negative control and were at par with that of commercial antibodies (Table 2).

Discussion

PMMoV is a serious viral disease that considerably affects the quality and quantity of capsicum production [4, 13, 34] and causes severe symptoms when infection takes place at young stage of crop [41]. Since 1984, the virus has been reported from different parts of capsicum growing areas of the world including India and Pakistan [3, 49], therefore the virus is distributed worldwide [33]. The symptoms produced by PMMoV sometimes remain unnoticed in the

Table 1 Evaluation of polyclonal antiserum produced against PMMoV-HP1 CP expressed in *E. coli* using purified expressed protein, infected and healthy plant samples through DAC-ELISA

Dilutions	Purified expressed protein (346 µg/ml)	Infected plant samples	Healthy plant samples	$OD_{Inf}/OD_{Healthy}$
1:100	2.610	1.372	0.340	4.0
1:500	2.371	0.713	0.237	3.0
1:1000	1.644	0.448	0.203	2.2
1:2000	1.288	0.197	0.123	1.6
1:4000	0.942	0.167	0.114	1.5
1:8000	0.901	0.122	0.112	1.1

Table 2 Validation of Polyclonal antiserum through DAC-ELISA

S. No.	Sample	Type of sample	Absorbance value at 405 nm	
			1:500	1:1000
1.	Sample-1	Leaf	1.555	0.952
2.	Sample-2		1.774	0.956
3.	Sample-3		1.722	0.976
4.	Sample-4		1.679	0.982
5.	Sample-5		1.456	1.129
6.	Sample-6		1.605	1.050
7.	Sample-7		1.714	0.959
8.	Sample-8		1.623	1.080
9.	Sample-9		1.488	0.973
10.	Sample-10		1.710	1.050
11.	Sample-11		1.730	1.008
12.	Sample-12		1.665	0.999
13.	Sample-13		1.669	1.060
14.	Sample-14		1.749	1.131
15.	Sample-15		1.733	1.066
16.	Sample-16		1.722	1.062
17.	Sample-17		2.532	1.382
18.	Sample-18		2.633	1.411
19.	Sample-19		1.790	0.805
20.	Sample-20		1.578	0.989
21.	CMV		0.345	0.162
22.	TMV		0.404	0.212
23.	TSWV		0.360	0.222
24.	TYLCV		0.277	0.209
25.	PVY		0.353	0.237
26.	PVMV		0.249	0.247
27.	Sample 21	Seed	1.031	0.905
28.	Sample 22		1.057	0.834
29.	Sample 23		0.987	0.512
30.	Sample 24		1.134	0.648

*DAC-ELISA from leaf tissue: Commercial Kit: 2.594, 2.435, Negative Control: 0.309

**DAC-ELISA from seed samples: Commercial Kit: 1.376, 1.326, Negative Control: 0.160

field till the fruit development [41] thus timely and accurate detection of the virus in field leads to the successful formulation of management strategies. Among various detection methods employed for plant virus disease diagnosis, ELISA has been the method of choice across the globe due to simplicity of the protocol, effectiveness and availability of the commercial kits for routine screening of large number of samples [40, 60].

The existence of 3 pathotypes viz., P_{12}, P_{123} and P_{1234} has been reported to infect capsicum in different parts of the world. Rialch et al. [45] determined the full genome sequence of PMMoV-HP1 and identified its pathotype P_{12} on the basis of amino acid sequence of CP from India. In the present study, the CP of the PMMoV-HP1 (P_{12}) was successfully amplified using RT-PCR and cloned into an expression vector, and polyclonal antiserum was raised against the expressed CP. Since CP is the most relevant viral protein used in immunodiagnostic detection procedures [15], thus this strategy has been used to produce antibodies against different plant viruses which are unstable or difficult to purify from infected host plant in sufficient quantity [23, 25, 29–31, 50]. The use of bacterially expressed protein as immunogen is preferred over the purified virus preparations from infected hosts. In some host plants the purification becomes difficult due to low yield of virion particles thus requires large quantity of infected tissue and may results in non-specific reaction because of mixed infection or host protein contamination [11]. *Capsicum* spp. is reported to being infected by 7 *Tobamovirus* species viz., TMV, *Tomato mosaic virus* (ToMV), *Tobacco mild green mosaic virus* (TMGMV), *Bell pepper mottle virus* (BPeMV), *Paprika mild mottle virus* (PaMMV), *Obuda pepper virus* (ObPV) and PMMoV [28]. Thus purified virus preparation from such hosts harboring multiple infections like *Capsicum* spp. may possess contamination of host proteins as well as different tobamoviruses which are difficult to distinguish on the basis of symptoms and virus morphology. With the advancement in virus research, now it has become possible to express the recombinant CP in bacterium and use as an antigen which is the best alternative route instead of purification directly from plant cells. The immunogen prepared through recombinant DNA technology remains free from any form of contamination thus provides pure antigen for antibody production [14]. The polyclonal antiserum generated through this method is comparatively more specific and sensitive as compared to those raised against purified virus particles [6].

In the current study, the PMMoV-CP was first cloned in pGEM-T easy cloning vector (Promega) and then subcloned in expression vector, pET-28a, to eliminate the instability of the plasmid due to the production of proteins potentially toxic to host cells [47]. The major protein band observed in the induced culture had higher molecular weight ($\sim$ 26 kDa) than that of observed in case of crude plant sap (17 kDa) because of the presence of 60 amino acids from the expression vector used including 6 Histidine amino acid residues at N-terminus. The solubility of the protein rely on the characteristics of the protein to be expressed (proteins with cysteine residue, improper formation of disulphide bond), nature of the host, the rate/level of expression (high or low), applied temperature and media composition [43]. In present study, the overexpression was induced by incubating the cells overnight at 16 °C which led to high level of expression which might have resulted in

formation of insoluble aggregates in *E. coli*; known as inclusion bodies.

As the recombinant protein had 6X Histidine tag at N-terminus, Ni–NTA columns were used to purify the target protein as histidine strongly binds to the immobilized Ni^{2+}-NTA matrices. The 6X Histidine residues have no immunogenic properties, thus tags were not removed from the purified target protein before injecting into the animal. The polyclonal antiserum raised in New Zealand White male rabbit was analyzed for its specificity and sensitivity to PMMoV through Western blot assay and DAC-ELISA.

The evaluation of raised antiserum through western blot assay and DAC-ELISA successfully detected PMMoV without any non-specific reaction. In the current study, almost at all the antiserum dilutions, a positive reaction was observed in case of purified protein sample as well as crude extract of PMMoV infected leaves in western blot assay. This confirmed that the antiserum raised was capable of detecting native CP from total proteins extracted from pepper plants infected with PMMoV and did not react with the plant proteins. There was a considerable difference in the molecular mass of the CP band observed in both cases as the CP in the purified expressed protein sample was fused with tags. The molecular mass of the band observed in case of infected plant tissue were of $\sim$ 17 kDa which is the molecular weight of PMMoV-CP. In DAC-ELISA dilution up to 1:1000 was able to clearly differentiate the infected samples from the healthy ones, while at dilution 1:8000 the absorbance value of both PMMoV infected and healthy sample were at par. So far, only two reports exists in the literature on production of antiserum against PMMoV using purified virus preparation [2] and expressed recombinant CP [32] as immunogen from Saudi Arabia and China, respectively. Afaf et al. [2] used the purified virus from infected plants as antigen and performed Tissue blot immuno binding assay (TBIA) and Dot blot immuno binding assay (DBIA) to evaluate the efficiency of antiserum. The virus titre observed by Afaf et al. [2] was 1/1024. The single purification protocol cannot be applied for different viruses, apparently similar viruses, or even different strains of a virus. Moreover, virus purification is more of an art than science. Additionally, to get more specific antiserum it is always recommended to use bacterially expressed viral gene which gives more consistent results due to its purity. The maximum titre of antiserum developed by Kun et al. [32] exhibiting positive reaction with antigen obtained from infected leaf tissue in Indirect-ELISA was 1:4000 which is 4 times higher than observed in our study, though the rate of positive samples was 38 per cent. Whereas in the present study, the rate of positive samples observed through DAC-ELISA was 100 per cent as all the samples from artificially inoculated plants tested positive using the antisera raised in the present study.

This is the first report of prokaryotic expression of PMMoV-CP (P_{12}) in pET-28a expression vector and production of polyclonal antiserum against CP of PMMoV-HP1, identified as P_{12} pathotype from India.

The specificity, sensibility and reproducibility of polyclonal antibodies raised in present study clearly indicate the utility of antibodies raised against CP region of PMMoV-HP1. Moreover the cross reactivity of antibodies raised against P_{12} pathotype need to be checked against other PMMoV strains, though we have not so far recorded the presence of P_1, P_{123} and P_{1234} pathotypes in our state. Further, the polyclonal antibodies developed in present study can be used for developing indigenous and low cost rapid immune-diagnostic strip which can be employed for detecting PMMoV infection in field itself without requiring any high cost instruments and expertise.

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Compliance with ethical standards

Conflict of interest There is no conflict of interest among the authors.

Human and animals rights The antiserum was produced in rabbits through custom hiring services from Genei Laboratories Pvt. Ltd. (Bangalore, India). All applicable national, and/or institutional guidelines for the care and use of animals were followed.

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